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These oscillations are thought to be the basis of bursting activity. In the presence of TTX, TRH also induced oscillations in membrane potential (Fig. 2C). The frequency of these oscillations was voltage-dependent, and the oscillations disappeared when the membrane was hyperpolarized. The voltage dependence of the oscillations was similar to that observed for rhythmic bursting (Fig. 2A), which suggests that the oscillations were responsible for the development of bursting activity. This experiment also showed that TRH affected NTS neurons in the absence of spike-induced transmitter release.

Thyrotropin-releasing hormone thus alters the activity in some NTS neurons from a nonrhythmic to a rhythmic pattern. The most straightforward explanation for our observations is that TRH acts directly on the membrane properties of NTS neurons, transforming them into endogenous burster neurons (*13*). Neurons that exhibit endogenous bursting behavior in the presence of neuroactive substances have been observed in the nervous systems of invertebrates (*14*) and are referred to as conditional bursters. The action of TRH on NTS neurons within the DRG supports the hypothesis that this neuropeptide takes part in the control of rhythmic breathing in mammals. Further experiments are required to assess the role of conditional bursting activity in this system and to determine the mechanism by which TRH modulates the membrane excitability of NTS neurons.

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2. Although most respiratory neurons in the DRG fire during inspiration, a limited number fire during expiration. In cats, about 5 percent of the respiratory neurons in the DRG are classified as expiratory [see Cohen in (*1*)]. In the DRG of guinea pigs, the number of expiratory neurons is about 20 percent (*7*). In addition, the DRG of both cats and guinea pigs contains second-order sensory neurons called pump units. These neurons convey information from lung stretch receptors.
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6. Our slicing procedure was based on that described by R. Llinas and M. Sugimori [*J. Physiol.* 305, 171 (1980)]. Briefly, guinea pigs were anesthetized with methoxyflurane and decapitated. The brainstem, with the cerebellum attached, was quickly removed and placed for 30 to 45 seconds in Ringer solution cooled to 0°C. After being cooled, the cerebellum was removed. The brainstem was cut into a block of tissue and glued with cyanoacrylate to a cooled Teflon block. Slices were cut with a Vibratome (Camden Instruments) while the tissue was submerged in cold Ringer solution (125 m*M* NaCl, 26 m*M* $NaHCO_3$, 6.2 m*M* KCl, 2.4 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, 1.25 m*M* $NaHPO_4$, and 10 m*M* glucose). All solutions were aerated with a gas mixture of 95 percent O_2 and 5 percent CO_2. Slices were viewed under a dissecting microscope. Glass microelectrodes were filled with 3*M* KCl and had resistances between 50 and 80 megohms.
7. G. B. Richerson and P. A. Getting, *Soc. Neurosci. Abstr.* 9, 1163 (1984). Guinea pigs were anesthetized with methoxyflurane and nitrous oxide, paralyzed with gallamine triethiodide, and artificially ventilated. The firing of NTS neurons was recorded extracellularly by means of glass microelectrodes. Fast green was ejected from the microelectrode to mark the recording site for later histological analysis. A cuff electrode was used to monitor phrenic nerve activity. Cycle-triggered histograms [see Cohen in (*1*)] were used to correlate the activity of respiratory neurons in the NTS with the phrenic nerve discharge. The types of respiratory neurons in the guinea pig were similar to those in the cat. The location of the DRG, however, was slightly more medial.
8. M. S. Dekin and P. A. Getting, *Brain Res.* 324, 180 (1984); *Fed. Proc. Fed. Am. Soc. Exp. Biol. Abstr.* 44, 1584 (1985).
9. After the brain slices had been exposed to TRH for a long period, a wash with normal Ringer solution for about 1 hour was required before another bursting response could be elicited. During the wash, individual action potentials gradually lost their DAP. The mechanism underlying the apparent desensitization and its removal was not studied in detail. The long periods of time required both to elicit a TRH response and to recover from desensitization prevented the determination of a dose-response relation for TRH in a single neuron.
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13. Although graded release of transmitter cannot be ruled out as a possible mechanism for rhythmic bursting, we feel that this is unlikely. A synaptic mechanism would be difficult to reconcile with the polarization (Fig. 2, A and C) and resetting (Fig. 2B) experiments.
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15. We thank D. Gallagher and J. Richerson for technical assistance and C. Edwards, J. L. Feldman, and D. R. McCrimmon for critical reading of the manuscript. Supported in part by NIH grants HL32336 and NS15350 (to P.A.G.), postdoctoral fellowship MH15172 (to M.S.D.), and predoctoral fellowship GM07337 (to G.B.R.).

11 February 1985; accepted 13 May 1985

Trans-Activator Gene of Human T-Lymphotropic Virus Type III (HTLV-III)

Abstract. *Human T-lymphotropic virus type III (HTLV-III) encodes a* trans*-acting factor that activates the expression of genes linked to the HTLV-III long terminal repeat. By functional mapping of complementary DNA transcripts of viral messenger RNA's the major functional domain of the gene encoding this factor was localized to a region immediately before the* env *gene of the virus, a region previously thought to be noncoding. This newly identified gene consists of three exons, and its transcription into messenger RNA involves two splicing events bringing together sequences from the 5' part (287 base pairs), middle (268 base pairs), and 3' part (1258 base pairs) of the HTLV-III genome. A similar messenger RNA with a truncated second exon (70 base pairs) does not encode a* trans*-acting function. It is proposed that this second messenger RNA is the transcript of a gene (3'-*orf*) located after the* env *gene. Messenger RNA's were also identified for the* env *and* gag-pol *genes of HTLV-III.*

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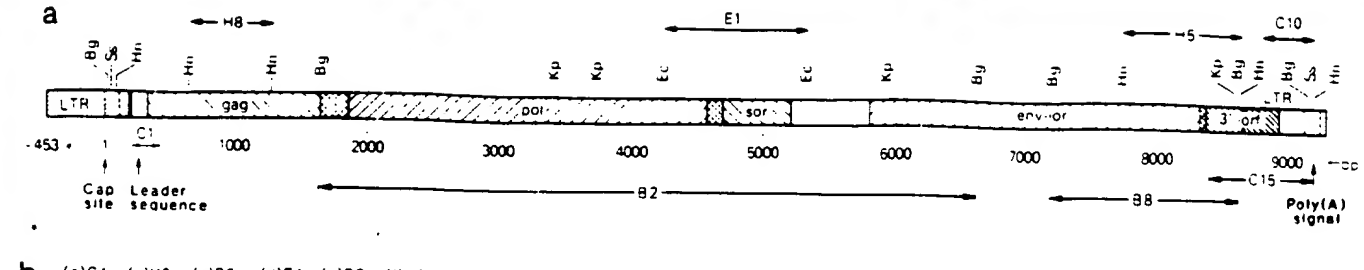
Human T-lymphotropic virus type-III (HTLV-III) is etiologically associated with the acquired immune deficiency syndrome (AIDS) (*1*, *2*). It belongs to the group of exogenous retroviruses called HTLV whose other members include HTLV-I and HTLV-II. HTLV-I has been etiologically linked to human adult T-cell leukemia-lymphoma (ATLL) (*3*, *4*), and HTLV-II, isolated originally from a patient with hairy cell leukemia (*5*), has not yet been linked to any human disease. These viruses share a number of biological and structural properties which include a tropism for $OKT4^+$ lymphocytes (*2*, *6*), the ability to induce giant multinucleated cells in vivo and in vitro (*2*, *7*), weak immunologic cross-reactivity of some virally encoded proteins (*8*), and distant nucleic acid sequence homologies (*9*, *10*). Despite these similarities, HTLV-III differs from HTLV-I and HTLV-II in many aspects of its structure and biology. For example, while infection of human T lymphocytes with HTLV-I or HTLV-II often results in transformation and immortalization (*3*, *4*), infection with HTLV-III generally leads to cell death (*1*, *2*).

The genomes of HTLV-III and related viruses have been molecularly cloned and sequenced (*10–14*), and five open reading frames (ORF's) have been identified (*11–15*) (Fig. 1). On the basis of the predicted amino acid sequence and alignment with known proteins of other retroviruses, it was postulated that the first, second, and fourth reading frames from the 5' end of the genome constituted the *gag*, *pol*, and *env* genes of HTLV-III. The third open reading frame, termed *sor*, has no correspondence in the



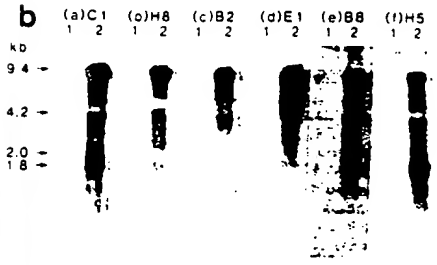


Fig. 1. Analysis of HTLV-III transcripts in virus-infected cells. Poly(A)-selected cellular RNA was hybridized with HTLV-III subgenomic probes by the Northern blot procedure (*28*). The derivation of the subgenomic probes, designated C1, H8, B2, E1, B8, and H5, along with a physical map of HTLV-III, is shown at the top. Lanes 1 and 2 in each panel are for uninfected and HTLV-III–infected H4 cells, respectively. Subgenomic probes were obtained by subcloning in plasmid vectors the fragments of HTLV-III genome generated by cleavage with specific restriction enzymes. The subclones were selected and characterized by restriction mapping, hybridization, and DNA sequencing. The restriction enzyme designations are Bg, Bgl II; Ss, Sst I; Hn, Hind III; Kp, Kpn I; and Ec, Eco RI.

HTLV-I or HTLV-II genome and its function is unknown. The fifth open reading frame (3′-*orf*) extends into the 3′ long terminal repeat (3′-LTR) and is truncated in some HTLV-III clones. HTLV-III–infected cells contain a *trans*-acting factor which activates the expression of LTR-linked genes (*16*). Similar factors in HTLV-I, HTLV-II, and bovine leukemia virus (BLV) are the products of a unique viral gene termed *tat* (also called *x-lor*) (*17*). On the basis of structural similarities of the predicted protein of 3′-portion of the unusually long *env* gene of HTLV-III with that of *tat* gene products, it was suggested that the *trans*-acting factor of HTLV-III is encoded by the 3′-portion of *env* gene. However, we show here that the major functional domain of the *trans*-activator gene of HTLV-III is located in what was previously thought to be a noncoding region between the *sor* and *env* genes. This gene consists of three exons and its transcription into a functional messenger RNA (mRNA) involves double splicing. We also describe the characteristics of the putative 3′-*orf* gene, which also consists of three exons, and identify putative mRNA's for *env* and *gag-pol* genes.

To identify *trans*-activator and other genes of HTLV-III, we took the direct approach of obtaining functional complementary DNA (cDNA) clones. In HTLV-III–infected cells there are at least four abundant virus-specific RNA's of 9.4, 4.2, 2.0, and 1.8 kilobases (kb) (*18*). To assess the genetic composition of the individual mRNA's and thus design strategies for obtaining specific cDNA clones, virus-specific RNA's were characterized further by Northern blotting with subgenomic viral DNA probes. As shown in Fig. 1, a 9.4-kb virus-specific RNA was detected by all the subgenomic probes. The ease of detection of other virus-specific RNA's was dependent on the probe used. All RNA species were scored by the probe designated C1, which was a cDNA clone containing leader sequences and a small 5′-part of the *gag* gene. The 4.2-kb RNA was detected by all the probes used in this study except H8, which gave a signal of only marginal relative intensity.

The 2.0-kb and 1.8-kb RNA's were easily scored by the probes representing the 3′-half of the viral genome (probes B8 and H5), and also but faintly by the probe B2. Additional hybridization of these RNA's with a leader sequence probe suggested that their synthesis involved at least one, probably two, splicing events. These results also suggested that the 4.2-kb mRNA was probably the *env* message and that the 9.4-kb RNA could be the genomic RNA as well as the viral readthrough message encoding *gag* proteins. Since the *pol* gene of HTLV-III is in a different reading frame from the *gag* gene, a spliced mRNA involving a frameshift must be synthesized to encode *pol*, but this may not be resolved from the unspliced genomic mRNA. If one assumes that the 3′-*orf* gene tran-

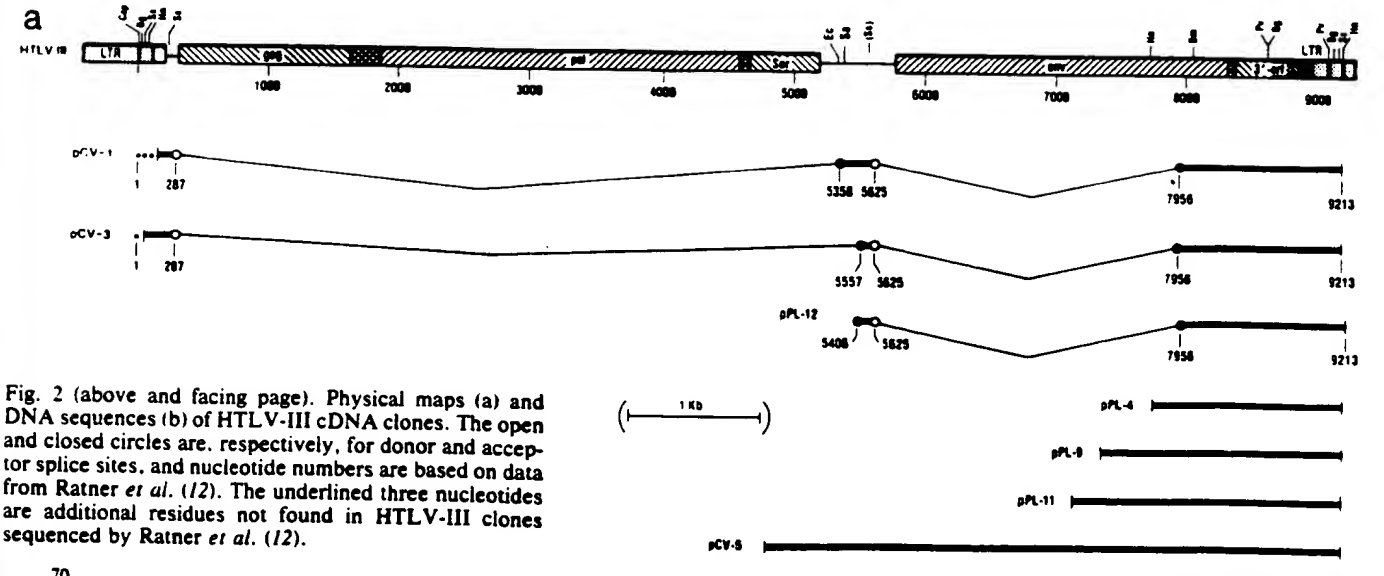
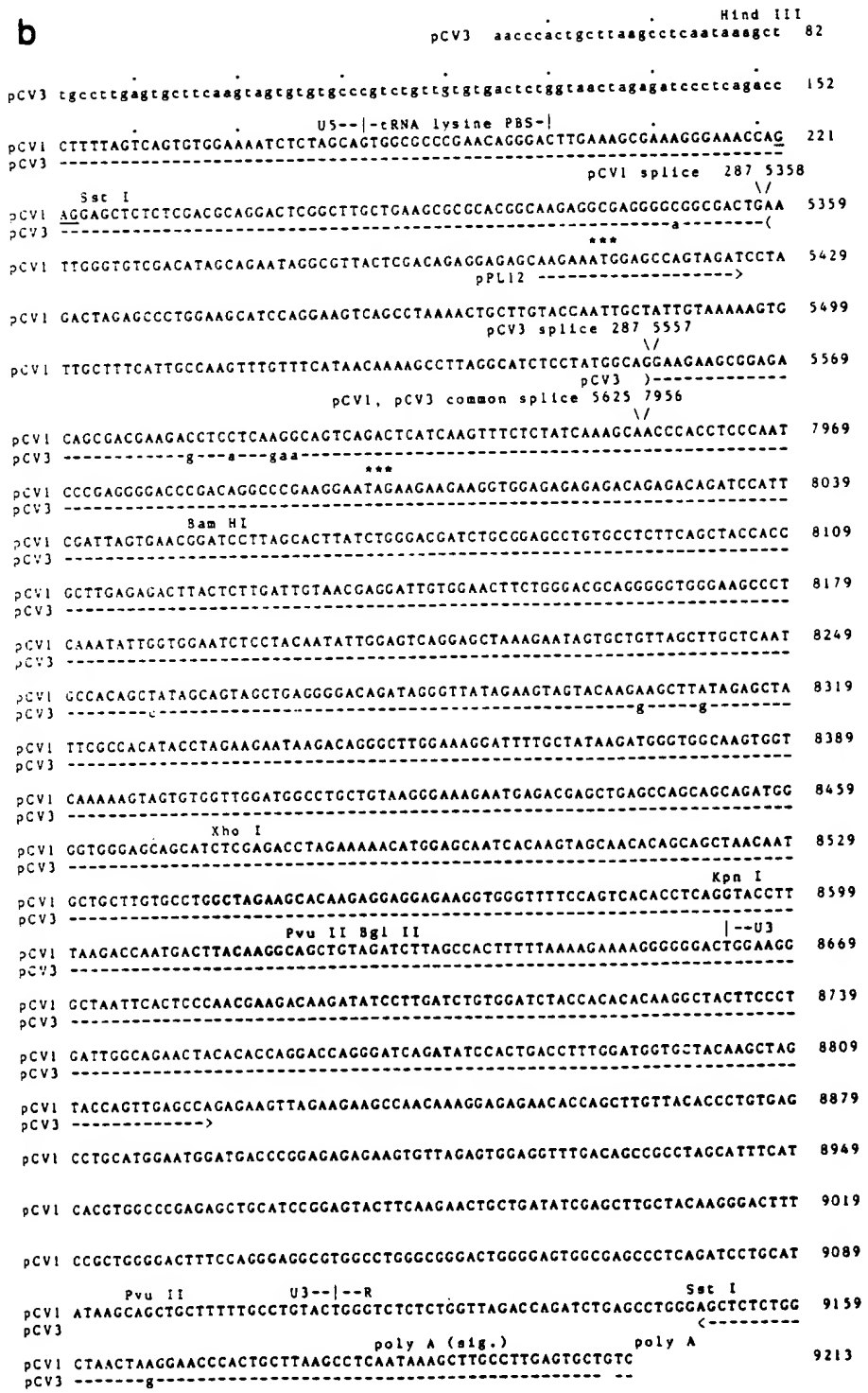


Fig. 2 (above and facing page). Physical maps (a) and DNA sequences (b) of HTLV-III cDNA clones. The open and closed circles are, respectively, for donor and acceptor splice sites, and nucleotide numbers are based on data from Ratner *et al.* (*12*). The underlined three nucleotides are additional residues not found in HTLV-III clones sequenced by Ratner *et al.* (*12*).

scribes abundant message or messages, either the 2.0-kb or 1.8-kb RNA, or both, are candidates for its transcripts. Similar observations were reported by Muesing *et al.* (*15*). By analogy to the mRNA of the *trans*-activating gene of HTLV-I and HTLV-II, which is also 2.0 kb in size, we speculated that one of the smaller mRNA's of HTLV-III encodes the *trans*-activating function.

We, therefore, screened cDNA libraries of RNA from HTLV-III–infected cells with probes C1 and B8, searching for clones with viral inserts of 2.0 kb or less. Two cDNA libraries were constructed for this purpose. One library was constructed in a mammalian expression vector (here termed pPL) containing SV40 regulatory sequences (*19*). The second library was constructed in a high-efficiency cloning vector lacking mammalian regulatory sequences (*20*), and the cDNA inserts of selected clones from this library were transferred to a second mammalian expression vector (*21, 22*), here termed pCV, which contained hy-



regulatory sequences (Fig. 3). Several selected cDNA clones from these libraries were characterized by restriction mapping and complete or partial DNA sequencing (Fig. 2).

It was clear that clones pCV-1 and pCV-3, each with about 1.8-kb inserts of viral sequences, corresponded to mRNA's whose synthesis involved two splicing events, consistent with our hybridization results. The pCV-1 message was apparently transcribed with the use of a donor splice site at nucleotide 287 and an acceptor splice site at nucleotide 5358, with the second donor and acceptor splice sites located at nucleotides 5625 and 7956, respectively (Fig. 2). Synthesis of the pCV-3 message used the same first donor and second acceptor and donor splice sites, but the first acceptor splice site was located at nucleotide 5557 (Fig. 2). In each case, the donor and acceptor splice site sequences were GT and CAG, respectively. The viral insert in clone pPL-12 contained sequences in common with clone pCV-1, utilizing the same second splice junction (Fig. 2), and appeared to be a partial transcript of the message similar or identical to that contained in clone pCV-1. The other cDNA clones shown in Fig. 2 were partial transcripts of viral genomic or subgenomic mRNA's as determined by restriction mapping and confirmed in some cases (for example, pCV-5) by direct DNA sequencing.

To ascertain which of these cDNA clones contained sequences with *trans*-activating function, we constructed a plasmid containing HTLV-III LTR sequences 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene (pC15CAT) (Fig. 3). It was shown previously that the LTR of HTLV-III can function as a promoter for the CAT gene in human lymphoid and other cells (*16*). The activity of the CAT gene in transfected cells can be conveniently measured and is correlated with steady-state CAT messenger levels (*23*). The cloned cDNA's were then cotransfected with pC15CAT DNA into human lymphoid H9 and JM cells by the DEAE-dextran protocol, and the CAT activity in the cytoplasm was measured. For representative results of such assays see Fig. 3; the data are compiled in Table 1. The results were reproducible within the experimental errors indicated (Table 1). Clones pCV-1 and pPL-12 clearly enhanced the CAT gene activity promoted by HTLV-III LTR as did the clone pCV-5. The other clones were consistently negative. The lack of enhancement by clones pCV-3 and pPL-11 compared with clones pCV-1 and pPL-12 could not be

Table 1. *Trans*-activating function of HTLV-III cDNA clones. The structures of many of the plasmid DNA's listed here are shown in Fig. 2. Others are pCV-0, vector pCV DNA without any insert; pCVHXb3, HTLV-III genomic clone HXb3 with cellular flanking sequences (*10*) inserted into the unique Xba I site of a derivative of the pCV vector (*22*); pSV_0-CAT, SV40CAT plasmid from which viral promoter has been deleted (*23*). Experiments were performed as described in the legend for Fig. 3.

Plasmid DNA	Relative activity of CAT gene	
	H9 cells	JM cells
pC15CAT	1	1
pC15CAT + pCV-0	0.9 ± 0.1*	
pC15CAT + pCV-1	23.6 ± 5.6†	90.4
pC15CAT + pCV-3	0.82 ± 0.12†	3.1
pC15CAT + pCV-5	7.3 ± 2.7*	16.8
pC15CAT + pPL-12	33.0 ± 6.5†	52.5
pC15CAT + pPL-4	1.0 ± 0.2*	0.6
pC15CAT + pPL-9	1.0 ± 0.2*	
pC15CAT + pPL-11	0.82 ± 0.26†	1.2
pC15CAT + pCVHXb3	16.7 ± 7.6†	48.9
pSV_0-CAT	1.10 ± 0.36†	0.6

*Average of two transfection assays. †Mean and standard deviation of four transfection assays.

attributed to differing transfection efficiencies, because the cells transfected with these DNA's contained equivalent levels of plasmid DNA's when analyzed by the Southern blot procedure. We also cotransfected cDNA clones with pRSV-CAT DNA, which contains Rous sarcoma virus LTR linked to CAT gene (*23*). None of the clones enhanced the CAT gene activity promoted by RSV LTR.

The structure-function analysis of cDNA clones used here allowed us to define the HTLV-III sequences responsible for its *trans*-activating function. Each of the clones pCV-1, pCV-3, and probably also clone pPL-12, consists of three exons (Fig. 2). Functional mRNA's of HTLV-III are synthesized by using two splicing events [see herein and (*16*)]. A double splicing mechanism for the synthesis of the *tat* mRNA has been suggested for HTLV-I (*17, 24*) as well as BLV (*25*) and may be a common property of the HTLV-BLV group of retroviruses. Comparison of the active clones pCV-1 and pPL-12 with the inactive clone pCV-3 suggests that the critical *trans*-activating functional domain is located in the second exon, which is truncated in clone pCV-3. It also suggests that the first and third exons are not sufficient for *trans*-activating function. The fact that clone pCV-5, and also pPL-12, both of which lack the first exon, are functionally active further suggests that the first exon is not necessary for gene activity. The lack of activity of clones that contain the third exon completely and exclusively, or nearly so (for example, pPL-4), suggests that the third exon by itself is not sufficient for *trans*-activating function. Further, all of the clones tested contained the complete sequence of the gene designated 3′-*orf*, which is located within the third exon; many of these clones were inactive. This suggests that the 3′-*orf* gene is not likely to be the *trans*-activator gene of HTLV-III. This is further supported by the fact that pCV-3, which contains a single ORF corresponding to 3′-*orf* and which probably represents the functional mRNA of this gene, is functionally inactive. The work of Sodroski *et al.* (*26*) confirms these observations and further demonstrates that entire deletion of the 3′-*orf* sequences does not affect *trans*-activat-

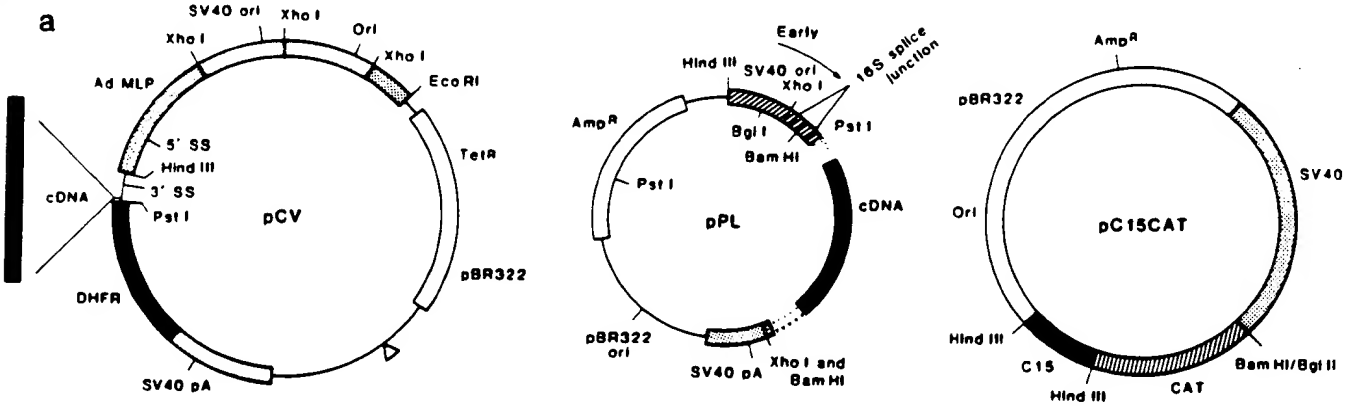


Fig. 3. Enhancement of HTLV-III LTR–promoted CAT gene expression by HTLV-III cDNA clones in human lymphoid H9 cells. (a) General structure of the mammalian expression vectors. The pCV contains duplicated SV40 replication origin (ori), adenovirus major late promoter (Ad MLP), splice sites (SS) from adenovirus and mouse immunoglobulin genes, mouse dihydrofolate reductase (DHFR) cDNA, and SV40 polyadenylation (pA) signal. (b) Analysis of CAT activity in transfected cells. About 1×10^7 H9 cells were washed with serum-free medium and incubated at 37°C for 1 hour in 5 ml of serum-free medium containing 50 m*M* tris-HCl (*p*H 7.3), DEAE-dextran (250 μg/ml), and 10 μg of plasmid DNA. The cells were then washed with medium containing 20 percent fetal bovine serum and incubated in 20 ml of serum-containing medium at 37°C. Forty-eight hours after transfection, cells were washed with phosphate-buffered saline and suspended in 100 μl of 0.25*M* tris-HCl (*p*H 7.4), and cellular extracts were prepared by three cycles of freezing (in ethanol and dry ice) and thawing (37°C). CAT activity was measured by incubating 20-μl aliquots of extracts with ^{14}C-labeled chloramphenicol (Cm) and 2.5 m*M* acetyl coenzyme A at 37°C overnight, and separating the acetylated chloramphenicol (AcCm) from the unacetylated form by ascending thin-layer chromatography. The chromatogram was autoradiographed and spots cut from the plate were then quantitated by scintillation counting. Lanes 1 to 8 are, respectively, for cells transfected with DNA's of (1) pSV_0CAT, (2) pRSVCAT, (3) pC15CAT, (4) pC15CAT plus pCVHXb3, (5) pC15CAT plus pCV-1, (6) pC15CAT plus pCV-3, (7) pC15CAT plus pPL-12, and (8) pC15CAT plus pPL-11.

ing function. The fact that clone pPL-12 is active and contains only part of the second exon narrows down the functional domain further and indicates that sequences between nucleotides 5357 and 5405 of the second exon are not necessary for *trans*-activating function.

A closer examination of the DNA sequences of active clones pCV-1 and pPL-12 revealed that they contained, in addition to *3'-orf*, an identical ORF of 258 bp consisting of 215 bp of the second exon and 43 bp of the third exon. This ORF starts with the initiator codon ATG located at position 5411 in the second exon and ends with the termination codon at position 7999 in the third exon (Fig. 4). It is absent in the inactive clone pCV-3, as this clone lacks the first 145 bp, including ATG. It is interesting that this ORF is well conserved among divergent HTLV-III isolates, suggesting the functional importance of this region. This ORF predicts a polypeptide of 86 amino acid residues (9 to 10 kD) rich in basic amino acids and with an uncommonly large number of prolines and arginines, and with no potential glycosylation sites. There is a striking cluster of lysine-arginine residues from amino acids 49 to 57. The hydrophilic nature of the predicted polypeptide (Fig. 4) and the absence of a hydrophobic signal peptide–like sequence at the NH_2-terminus suggest an intracellular localization of this protein. Its highly basic composition would be consistent with its being an intranuclear and DNA-binding protein. If the *trans*-activating function of HTLV-III is mediated by a protein, the results provide strong circumstantial evidence that the putative polypeptide may be the functional protein. Although the size and structure of this protein is different from the functionally analogous proteins of HTLV-I, HTLV-II, and BLV, its mechanism of action may be similar. Such proteins of HTLV-I and HTLV-II are located in the nucleus and probably act by direct binding to viral and possibly some cellular regulatory nucleotide sequences (*17, 27*). Since these proteins play a critical role in the transforming activity of HTLV-I and HTLV-II, it will be of interest to determine the role of this HTLV-III protein in the cytopathic activity of the virus.

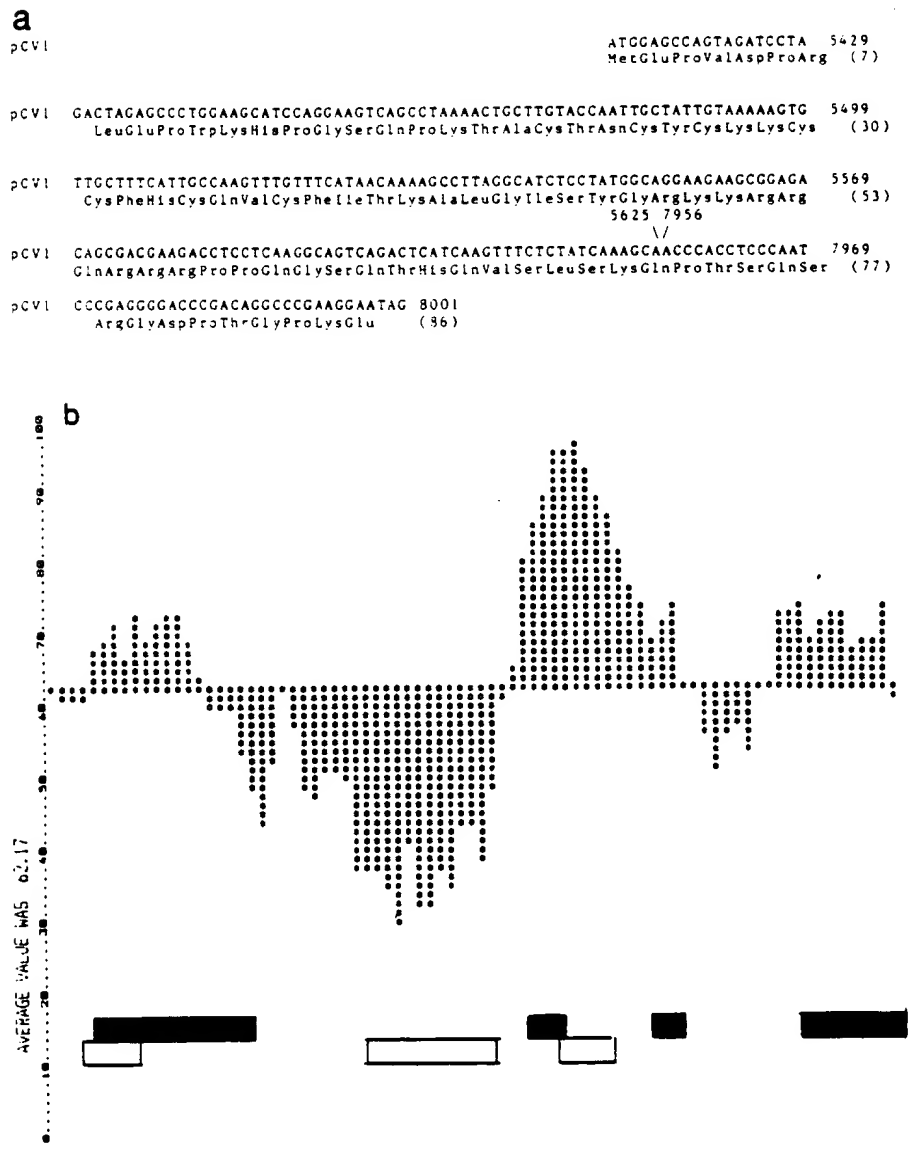


Fig. 4. Open reading frame found in cDNA clones pCV-1 and pPL-12. (a) Nucleotide and predicted amino acid sequence. (b) Hydrophilicity profile and predicted secondary structure of the putative polypeptide analyzed according to Kyte and Doolittle (*29*) and Chou and Fasman (*30*). Open and closed boxes represent α-helical structures and β-turns, respectively.

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